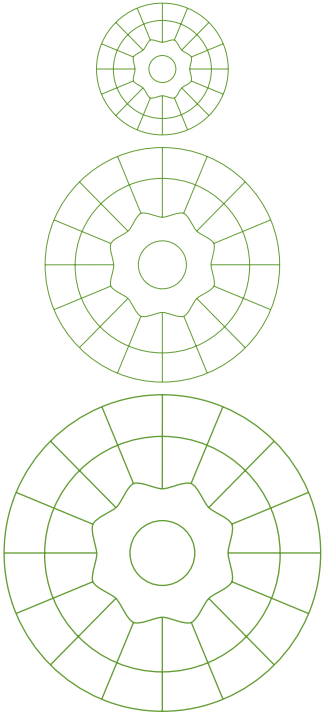
Duolink® In Situ

USER MANUAL – FLUORESCENCE



OLINK
BIOSCIENCE

The protocols in this manual are compatible with all Duolink In Situ PLA probes, Duolink In Situ Detection Reagents Green (Art. no. 92014), Orange (Art. no. 92007), Red (Art. no. 92008) and Far Red (Art. no. 92013)

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1. Introduction

Duolink® In Situ reagents from Olink® Bioscience enable detection, visualization and quantification of individual proteins, protein modifications and protein interactions in tissue and cell samples prepared for microscopy. The target is detected using one or two primary antibodies depending on the application. In the case that two primary antibodies are used, they must have been raised in different species.

The Duolink In Situ reagents are based on *in situ* PLA®, which is a proximity ligation assay technology. A pair of oligonucleotide labeled secondary antibodies (PLA probes) generates a signal only when the two PLA probes have bound in close proximity, either to the same primary antibody or two primary antibodies that have bound to the sample in close proximity. The signal from each detected pair of PLA probes is visualized as an individual fluorescent spot. These PLA signals can be quantified (counted) and assigned to a specific subcellular location based on microscopy images.

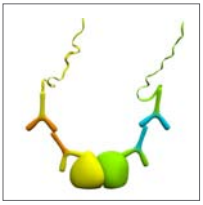
2. Principle of the assay

Typical starting materials are adherent cells, cytospin preparations or tissue sections on a glass slide, fixed, pre-treated and blocked with a blocking reagent according to the requirements of the primary antibodies used.

A. The samples are incubated with primary antibodies that bind to the protein(s) to be detected.

A.

B. Secondary antibodies conjugated with oligonucleotides (PLA probe MINUS and PLA probe PLUS) are added to the reaction and incubated.

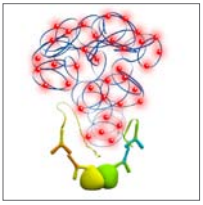


B.

C. The Ligation solution, consisting of two oligonucleotides (illustrated as red bands) and Ligase, is added and the oligonucleotides will hybridize to the two PLA probes and join to a closed circle if they are in close proximity.

C.

D. The Amplification solution, consisting of nucleotides (not shown) and fluorescently labeled oligonucleotides, is added together with Polymerase. The oligonucleotide arm of one of the PLA probes acts as a primer for a rolling-circle amplification (RCA) reaction using the ligated circle as a template, generating a concatemeric (repeated sequence) product. The fluorescently labeled oligonucleotides will hybridize to the RCA product. The signal is easily visible as a distinct fluorescent spot and analyzed by fluorescence microscopy.



D.

Fig 1. Assay principle.

3. Applications

3.1 DETECT AND QUANTIFY PROTEIN INTERACTIONS

Detection and quantification of interacting proteins can be done using two different primary antibodies against each of the two proteins of interest.

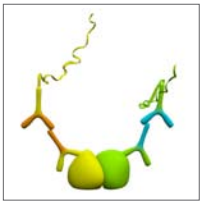


Fig 2. Protein interactions

3.2 DETECT AND QUANTIFY PROTEIN MODIFICATIONS

Detection and quantification of a protein and its specific post-translational modification can be done using two different primary antibodies, one directed against the target protein and one against a modification site on the same protein.

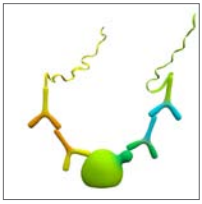


Fig 3. Protein modifications

3.3 DETECT AND QUANTIFY PROTEIN EXPRESSION

Detection and quantification of protein expression can be done with two different approaches.

(A) To detect and quantify one single protein with high sensitivity, use only one primary antibody.

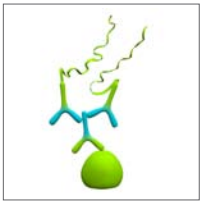


Fig 4. (A) Single recognition

(B) To detect and quantify one single protein with high specificity, use two different primary antibodies directed against two different epitopes on the same protein.

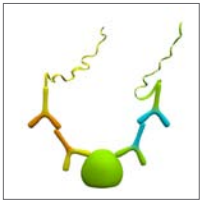


Fig 5. (B) Double recognition

4. Reagents and equipment

4.1 DUOLINK IN SITU REAGENTS

The Duolink In Situ reagents are generic reagents using secondary antibodies for detecting presence of analyte-specific primary antibodies provided by the user. To run a Duolink In Situ assay you need the following Duolink In Situ components.

- PLA probe MINUS
- PLA probe PLUS
- Detection Reagents
- Wash Buffers A and B
- Mounting Medium with DAPI

4.1.1 DUOLINK IN SITU PLA PROBES

The choice of PLA probes depends on the species of your primary antibodies and your application, see section 3. For a complete list of PLA probes, visit www.olink.com

Each Duolink In Situ PLA probe product contains the following:

- Blocking Solution – *For blocking of the sample if you have not already optimized your primary antibody with another blocking solution. Supplied in ready-to-use concentration*
- Antibody Diluent – *For dilution of PLA probes and in some cases the primary antibodies (section 5.4.3). Supplied in ready-to-use concentration*
- PLA probe (5x) stock – *Secondary antibody conjugated with a PLA oligonucleotide*

4.1.2 DUOLINK IN SITU DETECTION REAGENTS

Duolink In Situ Detection Reagents are available with four different fluorophores.

DETECTION REAGENTS	EXCITATION (nm)	EMISSION (nm)	FILTERS FOR IMAGE ACQUISITION
Detection Reagents Green	495	527	same filters as e.g. Cy2 or FITC
Detection Reagents Orange	554	579	same filters as e.g. Cy3
Detection Reagents Red	594	624	same filters as e.g. Texas Red
Detection Reagents Far Red	644	669	same filters as e.g. Cy5

Each Duolink In Situ Detection Reagents box contains the following:

- Ligation (5x) – *Contains oligonucleotides that hybridize to the PLA probes and all components needed for ligation except the Ligase*
- Ligase (1 U/µl)
- Amplification (5x) – *Contains all components needed for Rolling Circle Amplification except the Polymerase. Included are also oligonucleotide probes labeled with a fluorophore that hybridize to the RCA product*
- Polymerase (10 U/µl)

4.1.3 DUOLINK IN SITU MOUNTING MEDIUM WITH DAPI

Optimal mounting medium for preservation of the PLA signals. It also contains DAPI nuclear stain (excitation 360 nm and emission 460 nm). Aqueous mountant which does not solidify.

4.1.4 DUOLINK IN SITU WASH BUFFERS

Duolink In Situ Wash Buffers contains 3 pouches of Wash Buffer A and 1 pouch of Wash Buffer B, to be dissolved in high purity water.

4.2 REAGENTS TO BE SUPPLIED BY THE USER

- Reagents required for fixation and antigen retrieval of the sample (according to your own protocol, specific for each antigen and antibody used)
- Primary antibody or antibodies (matching a set of PLA probes)

4.3 EQUIPMENT NEEDED

- Fluorescence microscope equipped as follows:
  - Excitation/emission filters compatible with fluorophore and nuclear stain excitation/emission
  - Camera and software for image acquisition (optional)
- Staining jars
- Pen or mask for delimitation of reaction area
  (grease pen or silicon mask, e.g. ImmEdge™ Pen from Vector Laboratories)
- Shaker
- Humidity chamber (moist chamber)
- Freeze block for enzymes
- Incubator, +37°C
- Pipettes (covering the range from 1 µl to 1000 µl)
- Cover slips compatible with fluorescence microscopy
- High purity water (sterile filtered, MilliQ® or similar)

4.4 DUOLINK IMAGETOOL – SOFTWARE FOR IMAGE ANALYSIS

The result from a Duolink In Situ experiment is typically a number of distinct fluorescent spots, PLA signals, of sub-micrometer size in various locations of the studied cells.
Use Duolink ImageTool to obtain objective quantification of PLA signals. The nuclei are automatically detected and cytoplasm size estimated, enabling single cell statistical analysis of expression levels in tissue or cell populations. Furthermore, regions of interest can be defined, a feature of particular relevance when studying tissue samples. Raw imaging data can be imported directly from the four major microscope vendors (Olympus, Leica, Nikon and Zeiss). The results data can be exported into an Excel sheet for further evaluation.

5. Assay considerations

5.1 CHOICE OF PRIMARY ANTIBODIES

The Duolink In Situ reagents are generic reagents using secondary antibodies for detecting presence of analyte-specific primary antibodies. Your choice of primary antibodies is crucial when setting up the PLA assay.

The primary antibodies should be of IgG-class, specific for the target to be detected and preferably affinity purified. The primary antibodies could be either polyclonal or monoclonal. To maximize your success rate, choose antibodies that are IHC and/or IF classified and follow the optimization guidelines given in section 5.2. Primary antibodies could also be qualified individually in a Duolink In Situ single recognition experiment (section 5.1.1) before a double recognition assay.

5.1.1 PRIMARY ANTIBODIES FOR DETECTION OF PROTEIN EXPRESSION

When single protein targets are to be detected you can use either one or two primary antibodies against your target.

A) One primary antibody (single recognition)

Usage of one primary antibody gives you an easy-to-set-up assay with high sensitivity. In this case only one antibody needs to bind the target under the conditions used (fixation, retrieval, buffer etc.). Using one primary antibody is recommended for detection of single target proteins only when a well performing specific primary antibody is available. If you use an unspecific antibody you will see high background due to the sensitivity of the assay. Using only one primary antibody is more efficient than using two primary antibodies when detecting low abundant targets.

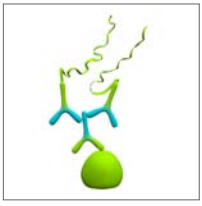


Fig 6. (A) Single recognition

B) Two primary antibodies (double recognition)

Usage of two primary antibodies will give you an assay with superior specificity.

When using two primary antibodies, they must be directed against different, non-competing epitopes on the same target molecule. The two primary antibodies must have been raised in different species. Also, both primary antibodies must bind to the target under the same conditions (fixation, retrieval, buffer etc.).

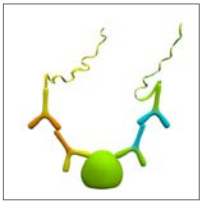


Fig 7. (B) Double recognition

5.1.2 PRIMARY ANTIBODIES FOR DETECTION OF PROTEIN MODIFICATIONS

Detection of protein modifications, such as phosphorylations, often suffers from low specificity. Our recommended strategy is to use two primary antibodies, one against the target protein and one against a modification site on the same protein. Preferably a specific modification site is targeted, but it is also possible to use a generic antibody against a modification site. Using two primary antibodies will give you superior specificity for your modification assay compared to using one single primary antibody. The two primary antibodies must be raised in different species. Also, both primary antibodies must bind to the target under the same conditions (fixation, retrieval, buffer etc.).

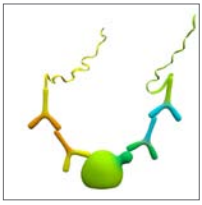


Fig 8. Protein modifications

5.1.3 PRIMARY ANTIBODIES FOR DETECTION OF PROTEIN INTERACTIONS

Duolink In Situ provides an excellent way to detect protein interactions. This is done using two primary antibodies, each directed against one of the targets of interest. The two primary antibodies must be raised in different species and must bind to the target under the same conditions (fixation, retrieval, buffer etc.).

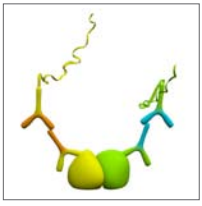


Fig 9. Protein interactions

5.2 PRIMARY ANTIBODY OPTIMIZATION

You will save time and increase your success rate if you optimize your primary antibodies to be sure that they work properly in your material.

The conditions for your primary antibodies should be optimized with respect to:

- Sample fixation
- Antigen retrieval
- Blocking solution
- Antibody diluent
- Primary antibody concentration

Sample pre-treatment for Duolink In Situ is identical to procedures used for immunohistochemical (IHC) and/or immunofluorescence (IF) staining. If you already have a working assay for IHC or IF, use the same pre-treatment protocol. The primary antibody concentration may need to be titrated and optimized for the Duolink In Situ conditions.

When using two primary antibodies, optimization is best done for one primary antibody at the time using the Duolink In Situ single recognition approach. These conditions can then be used as a start for optimizing your assays using both primary antibodies in the same Duolink In Situ assay.

When using two primary antibodies it may in some cases be difficult to find conditions compatible with both of the selected primary antibodies. Under these circumstances it is advisable to search for other antibodies recognizing alternative epitopes.

For further advice on immunostaining optimization we recommend the Education Guide from Dako (http://pri.dako.com/08002_25may06_ihc_guide_book.pdf).

5.3 SAMPLE TYPE

Duolink In Situ reagents can be used with a broad range of sample types depending on the preferences of your primary antibodies. Duolink In Situ has so far shown to be successful with the following sample types.

- Adherent cell cultures
- Cytospin preparations
- Formalin fixed paraffin embedded cell samples
- Formalin fixed paraffin embedded tissue sections
- Frozen tissue sections
- Fresh tissue sections

5.4 PRE-TREATMENT

Before you start, your samples should be deposited on glass slides and sufficiently pre-treated with respect to fixation, retrieval and permeabilization. It is crucial for the performance of the assay to optimize the conditions for the primary antibodies. Also, the choice of wash buffer may be dependent on your primary antibodies. Use the recommendations from the vendor of your primary antibodies, if available. If you are using chamber slides or cover slips, follow the guidelines in Appendix B or C.

5.4.1 FIXATION

Different fixations will influence the antigenicity and thus the performance of your primary antibodies. If there is no fixation recommendation for your antibody you must optimize this for your application.

For cell materials like cells grown on slides or cytospin preparations you should choose the fixation most suitable for your primary antibody. The best choice of fixation may vary depending on your cell material, antibody and the type of protein you want to study. Duolink In Situ is compatible with all fixations typically used for IHC including:

- Ethanol
- Acetone
- Paraformaldehyde (PFA)
- Zinc
- Formalin

5.4.2 ANTIGEN/EPITOPE RETRIEVAL

Antigen/epitope retrieval is mostly used for formalin fixed paraffin embedded (FFPE) material. Formalin fixation is masking the epitopes which then can be un-masked by antigen/epitope retrieval. Different methods for antigen/epitope retrieval will influence the performance of your primary antibodies and must be optimized for your application. Duolink In Situ is compatible with all antigen retrieval methods typically used for IHC including:

- Heat Induced Epitope Retrieval (HIER)
- Enzyme treatment with Proteinase K, Pepsin, Trypsin etc.

For HIER it might be necessary to try a few different solutions of various pH.

5.4.3 BLOCKING SOLUTION AND ANTIBODY DILUENT

It is essential to use a proper blocking solution and antibody diluent. Use the recommendations from the vendor of your primary antibodies, if available. If you have previously optimized your assay in for example IHC, use the same conditions for Duolink In Situ.

There are two different protocols depending on whether you use your own blocking solution and antibody diluent or the Duolink In Situ Blocking Solution and Antibody Diluent.

PLA probe protocol – CUSTOM solutions

- Use this protocol if you use your own blocking and antibody diluent (recommended), see section 7.1

The same agent used for blocking of the sample should be present also in the antibody diluent, preferably together with some detergent to reduce background staining, as well as in the diluent for the PLA probes.

Note: *Ensure that you do not use bulk IgG from the same species as your primary antibodies as blocking reagent as this will cause false signals from the PLA probes.*

PLA probe protocol – Duolink In Situ Solutions

- Use this protocol if you have no previous experience, or no recommendation from the antibody vendor, regarding blocking and antibody diluents, see section 7.2

Note: *The Duolink In Situ Antibody Diluent contains salt, blocking agent and detergents. All to prevent unspecific binding of the antibodies. It is important that you first verify the function of your primary antibodies, one at the time, using Duolink In Situ single recognition or possibly IF or IHC. Use proper controls to verify that your individual antibodies do bind the correct target under the conditions used.*

5.5 CONTROLS

To be able to properly evaluate your results it is advisable to include both biological and technical controls. Examples of suitable controls are given below.

5.5.1 POSITIVE CONTROL

Ideally you should include a cell or tissue type that you know contains your target protein and that your primary antibodies should bind to. If you are using two primary antibodies, the primary antibodies should be known to bind in close proximity. This also enables you to verify the Duolink In Situ procedure.

5.5.2 NEGATIVE CONTROL

Ideally you should include a negative control with a cell line or tissue that does not express one or both of your targets. Such a control will give you information on the specificity of your primary antibodies. If this control gives you background signals in excess of signal obtained from omitting the primary antibody/antibodies, it means that your primary antibodies, under the present conditions, are binding to other targets than expected.

If facing problems with signals in your negative cell line/tissue you might need to titrate your primary antibodies or look for more specific antibodies.

5.5.3 NEGATIVE CONTROL, TECHNICAL

By omitting primary antibodies you will get a hint of how the PLA probe background looks like in your system. Alternatively, if you are using an approach with two primary antibodies, you can also choose to omit only one of your primary antibodies.

If facing problems with background signals with the primary antibodies omitted, consult the troubleshooting guide, see section 9.

6. Reagent preparation

6.1 DUOLINK IN SITU REAGENTS

Some Duolink In Situ reagents are supplied as concentrated stocks. Dilute required volumes of the stocks.

Note: *Do not store diluted reagents.*

Blocking Solution – store at +4°C

- Vortex before use
- Ready-to-use blocking solution, one drop equals approximately 40 µl

Antibody Diluent – store at +4°C

- Vortex before use
- For dilution of primary antibodies and PLA probes

PLA probe (5x) – store at +4°C

- Vortex before use
- Dilute 1:5 in Antibody Diluent or custom diluent immediately before use and vortex the solution before addition to the sample

Ligation (5x) – store at -20°C

- Thaw at room temperature and vortex before use
- Dilute 1:5 in high purity water immediately before use

  Note: *The buffer contains DTT that may precipitate during freezing. Vortex to dissolve/homogenize.*

Ligase (1 U/µl) – store at -20°C

- The Ligase should be kept at -20°C at all times. Use a freezing block (-20°C) when removing the enzyme from the freezer
- Add the Ligase to the reaction mix at a 1:40 dilution immediately before addition to the sample
- Ensure that the Ligation-Ligase reaction solution is thoroughly vortexed before addition to the sample

Amplification (5x) – store at -20°C

- Thaw at room temperature and vortex before use
- Dilute 1:5 in high purity water immediately before use

Polymerase (10 U/µl) – store at -20°C

- The Polymerase should be kept at -20°C at all times. Use a freezing block when removing the enzyme from the freezer.
- Add the Polymerase to the reaction mix at a 1:80 dilution immediately before addition to the sample
- Ensure that the Amplification-Polymerase reaction solution is thoroughly vortexed before addition to the sample

6.2 DUOLINK IN SITU WASH BUFFERS

The washing steps should be performed in a staining jar, with a minimum volume of 70 ml, on a shaker (cradle) with gentle orbital shaking. Washing after the primary antibodies should be performed in Duolink In Situ Wash Buffer A or in the wash buffer optimal for your primary antibodies. In all subsequent wash steps ensure to use Duolink In Situ Wash Buffers.

Wash Buffer A – store at room temperature

- To prepare a 1x buffer, dissolve the content of one pouch in high purity water to a final volume of 1000 ml
- Store pouches at room temperature. 1x solutions may be kept at room temperature for short time storage (one week or less). For long time storage store at +4°C. Bring the solutions to room temperature before use
- Alternatively make your own Duolink In Situ Wash Buffer A according to appendix A

Wash Buffer B – store at room temperature

- To prepare a 1x buffer, dissolve the content of one pouch in high purity water to a final volume of 1000 ml
- Store pouches at room temperature. 1x solutions may be kept at room temperature for short time storage (one week or less). For long time storage store at +4°C. Bring the solutions to room temperature before use
- For the final wash step, prepare 0.01x Wash Buffer B by diluting 1x buffer 1:100 in high purity water
- Alternatively make your own Duolink In Situ Wash Buffer B according to appendix A

6.3 SAMPLES

Before you start the Duolink In Situ protocol, the sample should have been deposited on a glass slide, sufficiently pre-treated to fit your primary antibodies with respect to fixation, retrieval and permeabilization, see section 5.2. Your reaction area must be delimited, with e.g. a grease pen or silicon mask. If you are using chamber slides or cover slips, follow the guidelines in Appendix B or C.

6.4 REACTION VOLUME

- Use open droplet reactions
- Perform the incubations *without a cover slip*
- Perform all incubations in a pre-heated humidity chamber
- Use volumes corresponding to your delimited reaction area. Never use less than 15 µl of total reaction volume, see Table 1 or Reaction Volume Guide (Art. no. 80520)

  Note: *It is important that all incubations are performed in a humid environment to prevent excessive evaporation. If the sample goes dry, this will give rise to severe artifacts.*

Table 1. Suitable reaction volume for different reaction areas.

AREA	TOTAL REACTION VOLUME
0.2 cm^2	15 µl
1 cm^2	40 µl
2 cm^2	80 µl
3 cm^2	120 µl
4 cm^2	160 µl
6 cm^2	240 µl
8 cm^2	320 µl
10 cm^2	400 µl

7. Assay protocol

7.1 PLA PROBE PROTOCOL – CUSTOM SOLUTIONS

Follow this protocol if you use your own blocking solution and antibody diluent that you know work with your primary antibodies.

Before you start, your samples should be deposited on glass slides and pre-treated with respect to fixation, retrieval and/or permeabilization. If you are using chamber slides or cover slips, follow the guidelines in Appendix B or C.

Use open droplet reactions *without a cover slip* and perform all incubations in a humidity chamber. Use volumes corresponding to your delimited reaction area, see the Reaction Volume Guide (Art. no. 80520) or section 6.4.

1.	**BLOCKING**
	Use your previously tested blocking solution.
a)	Add blocking solution to each sample.
b)	Incubate the slides.
2.	**PRIMARY ANTIBODIES**
	Use your previously tested buffer to dilute your primary antibodies.
a)	Dilute your primary antibody to a suitable concentration in your custom antibody diluent. If using two primary antibodies dilute the two antibodies in the same diluent.
b)	Tap off the blocking solution from the slides. Try to obtain an equal residual volume on each slide as this will affect reproducibility. *Do not allow the samples to dry before adding the primary antibodies as this will cause background.*
c)	Add the primary antibody solution to each sample.
d)	Incubate in a humidity chamber. *Use temperature and time optimal for your primary antibodies.*
3.	**PLA PROBES**
	Use the same buffer as for your primary antibodies or use Duolink In Situ Antibody Diluent. Note: *The buffer should contain the blocking agent that was used for blocking the samples.*
a)	Mix and dilute the two PLA probes 1:5 in your chosen buffer. Allow the mixture to sit for 20 min at room temperature. *E.g. for a 40 µl reaction take 8 µl of PLA probe MINUS stock, 8 µl of PLA probe PLUS stock and 24 µl of the antibody buffer.*
b)	Tap off the primary antibody solution from the slides.
c)	Wash the slides in a wash buffer suitable for your primary antibodies. *Washing should be performed in a staining jar, with a minimum volume of 70 ml on a shaker (cradle) with gentle orbital shaking. Bring the wash buffers to room temperature before use.*
d)	Add the diluted PLA probe solution.
e)	Incubate the slides in a pre-heated humidity chamber for 1 h at +37°C.

›› *Proceed to the Detection protocol in section 7.3 on page 18.*

7.2 PLA PROBE PROTOCOL – DUOLINK IN SITU SOLUTIONS

Follow this protocol if you use the Duolink In Situ Blocking Solution for blocking of your sample and Duolink In Situ Antibody Diluent for dilution of your primary antibodies.

Before you start, your samples should be deposited on glass slides and pre-treated with respect to fixation, retrieval and/or permeabilization. If you are using chamber slides or cover slips, follow the guidelines in Appendix B or C.

Use open droplet reactions *without a cover slip* and perform all incubations in a humidity chamber. The volume examples are based on 40 µl reaction volume suitable for 1 cm^2. Use volumes corresponding to your delimited reaction area, see the Reaction Volume Guide (Art. no. 80520) or section 6.4.

1.	BLOCKING
a)	Add one drop of Blocking Solution per 1 cm^2. *Ensure to cover the entire reaction area with Blocking Solution.*
b)	Incubate the slides in a pre-heated humidity chamber for 30 min at +37°C.

2.	PRIMARY ANTIBODIES
a)	Dilute your primary antibody or antibodies to suitable concentration in the Antibody Diluent.
b)	Tap off the Blocking Solution from the slides. Try to obtain an equal residual volume on each slide as this will affect reproducibility. *Do not allow the samples to dry before adding the primary antibodies as this will cause background.*
c)	Add the primary antibody solution to each sample.
d)	Incubate in a humidity chamber. *Use the optimal incubation temperature and time for your primary antibodies.*

3.	PLA PROBES
a)	Dilute the two PLA probes 1:5 in Antibody Diluent. *E.g. for a 40 µl reaction take 8 µl of PLA probe MINUS stock, 8 µl of PLA probe PLUS stock and 24 µl of Antibody Diluent.*
b)	Tap off the primary antibody solution from the slides.
c)	Wash the slides in 1x Wash Buffer A or in a wash buffer suitable for your primary antibody, preferably 2 x 5 min. *Washing should be performed in a staining jar, with a minimum volume of 70 ml on a shaker (cradle) with gentle orbital shaking. Bring the wash buffers to room temperature before use.*
d)	Add the PLA probe solution.
e)	Incubate the slides in a pre-heated humidity chamber for 1 h at +37°C.

›› *Proceed to the Detection protocol in section 7.3 on page 18.*

7.3 DETECTION PROTOCOL

Proceed from step 3e of the PLA probe protocols. Use open droplet reactions *without a cover slip* and perform all incubations in a humidity chamber. The volume examples are based on 40 µl reaction volume, suitable for 1 cm^2 reaction area. Adjust the volumes corresponding to your specific delimited reaction area, see the Reaction Volume Guide (Art. no. 80520) or section 6.4.

4. LIGATION

a) Dilute the Ligation stock 1:5 in high purity water and mix.
Wait to add the Ligase until immediately before addition to the samples. Take the addition of Ligase into account when calculating the amount of water added.
E.g. for a 40 µl reaction take 8 µl of the 5x Ligation stock and 31 µl of high purity water.

b) Tap off the PLA probe solution from the slides.

c) Wash the slides in 1x Wash Buffer A for 2 x 5 min under gentle agitation.

d) Remove the Ligase from the freezer using a freezing block (-20°C).
Add Ligase to the Ligation solution from step a) at a *1:40* dilution and vortex.
E.g. for a 40 µl reaction add 1µl of Ligase to 39 µl of Ligation solution.

e) Add the Ligation-Ligase solution to each sample.

f) Incubate the slides in a pre-heated humidity chamber for 30 min at +37°C.

5. AMPLIFICATION

Note: *Light sensitive reagents.*

a) Dilute the Amplification stock 1:5 in high purity water and mix.
Wait to add the Polymerase until immediately before addition to the sample. Take the addition of Polymerase into account when calculating the amount of water added.
E.g. for a 40 µl reaction take 8 µl of the 5x Amplification stock and 31.5 µl of high purity water.

b) Tap off the Ligation-Ligase solution from the slides.

c) Wash the slides in 1x Wash Buffer A for 2 x 2 min under gentle agitation.
Tap off all wash solution after the last washing.

d) Remove the Polymerase from the freezer using a freezing block (-20°C).
Add Polymerase to the Amplification solution from step a) at a *1:80* dilution and vortex.
E.g. for a 40 µl reaction add 0.5 µl of Polymerase to 39.5 µl of Amplification solution.

e) Add the Amplification-Polymerase solution to each sample.

f) Incubate the slides in a pre-heated humidity chamber for 100 min at +37°C.

6. FINAL WASH STEP

Note: *Light sensitive reagents. Wash and dry the slides protected from light. Ensure to have 1x and 0.01x Wash Buffer B available.*

a) Tap off the Amplification-Polymerase solution from the slides.

b) Wash the slides in 1x Wash Buffer B for 2 x 10 min.

c) Wash the slides in 0.01x Wash Buffer B for 1 min.

d) Let the slides dry at room temperature in the dark.

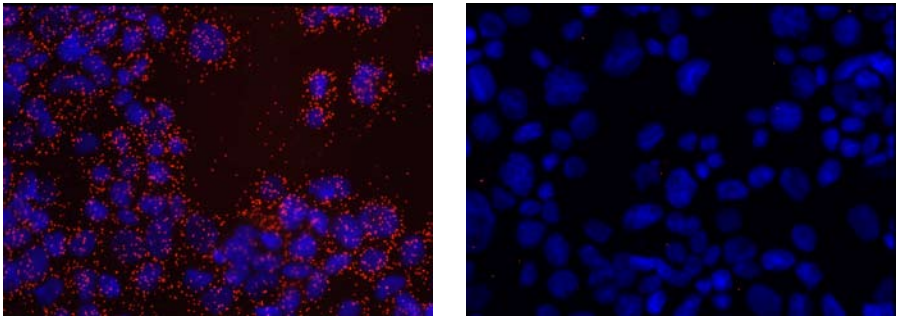
7. PREPARATION FOR IMAGING

Mount your slides with a cover slip using a minimal volume of Duolink In Situ Mounting Medium with DAPI, ensuring no air bubbles get caught under the cover slip. Nail polish can be used to seal the edges. Wait for approximately 15 min before analyzing in a fluorescence or confocal microscope, using at least a 20x objective. After imaging, store the slides at -20°C in the dark. *Duolink In Situ Mounting Media with DAPI is aqueous and does not solidify.*

8. Results

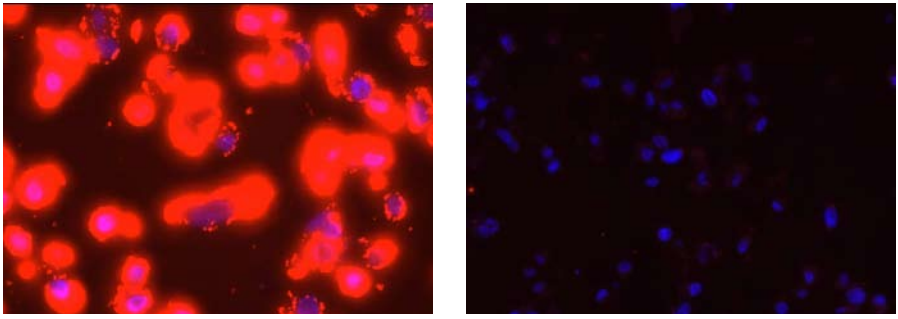
8.1 TYPICAL RESULTS

The result from a Duolink In Situ experiment is typically a number of discrete fluorescent spots, PLA signals, in various locations of the studied cells, see Figure 10. In some cases, when studying highly expressed proteins, the density of signals may be so high that the signals coalesce, see Figure 11.



A B

Fig 10. Detection of EGFR in cytospin preparations of A431 cells using Duolink In Situ with one primary antibody where the primary antibody has been titrated to a very low concentration to give individual signals. The pictures show a maximum intensity projection of the raw image based on 20 z-planes. PLA signals are shown in red and the nuclei in blue. The nucleus image was acquired in one z-plane.
A) Positive reaction. B) Negative control without primary antibodies.



A B

Fig 11. Detection of Her2 in FFPE preparations of SKBR-3 high expression cells using Duolink In Situ with two primary antibodies. In this case the antibodies have been titrated to give signals in low expression cell lines. The pictures show a maximum intensity projection of the raw image based on 20 z-planes. PLA signals are shown in red and the nuclei in blue. The nucleus image was acquired in one z-plane. A) Positive reaction. B) Negative control without primary antibodies.

8.2 IMAGE ACQUISITION

The PLA signal is recognized as a fluorescent spot, see Figure 10, in a fluorescence microscope using the appropriate filters for the detection fluorophore used. An individual signal is of sub-micrometer size.

For images taken in one focal plane, several signals can be either above or below the current focus. A true PLA signal is easy to "go through" by changing the focus making it appear and disappear. This does not apply to coalesced signals which may occur for highly expressed proteins, see below. To detect all PLA signals it is thus necessary to obtain images throughout the entire thickness of the sample. However, you may acquire images in one plane as long as all images to be compared are acquired in a similar position within the sample.

As with all fluorescent imaging it is important to keep settings constant during an experiment with regard to exposure time and filters used etc. It is recommended to get a feeling for the setting by use of one acquisition time needed for your specific microscope setting by use of one positive slide and one negative slide. For proper positive and negative controls please refer to section 5.5 Controls on page 13. It is important to remember that a negative slide without primary antibodies but with PLA probes is likely to contain a few signals (one or two signals every tenth cell) and therefore the image from the negative reaction should not necessarily be completely blank. The same setting should be used for all the images. If the number of PLA signals is large, it could happen that they coalesce all over the image or in certain cells. Care must then be taken to choose for how to set the acquisition time, whether to show the heavily expressed areas or to show all PLA signals, even individual ones.

8.3 DUOLINK IMAGETOOL – IMAGE ANALYSIS

To analyze the results, use Duolink ImageTool to obtain objective quantification of PLA signals. By using the software it is possible to obtain either the number of signals and cells per image, allowing average measurements, or to allocate each individual signal to a specific cell using single cell analysis. The nuclei are automatically detected and cytoplasm size estimated, enabling single cell statistical analysis of expression levels in tissue or cell populations.

A result can typically look as shown in Figure 12.

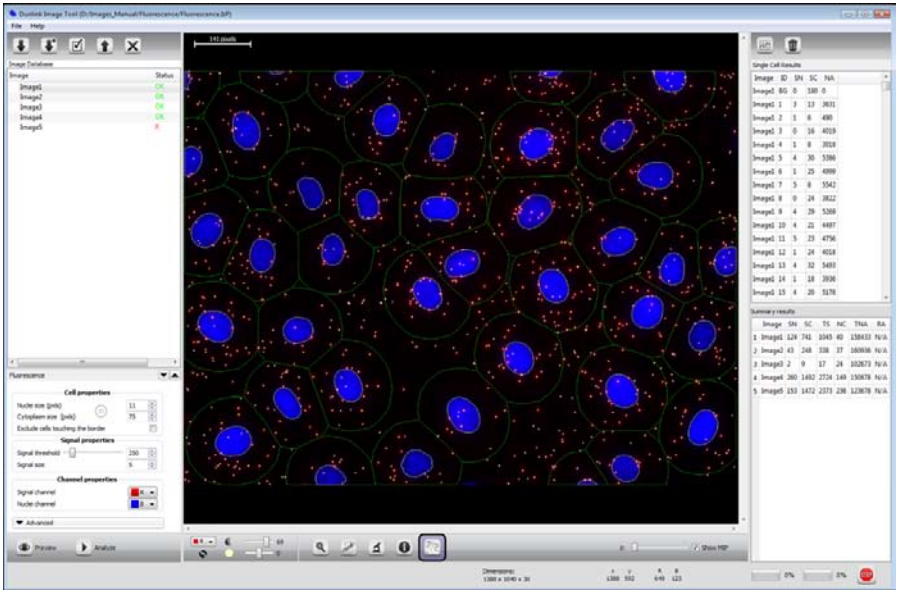


Fig 12. Analysis of an image from a fluorescence microscope.

Nuclei are blue, the red spots are the PLA signals representing the protein target of interest. PLA signals marked with white circles and nuclei outlined in yellow are quantified at analysis. The green outlines represent the user defined cytoplasm size.

When studying highly expressed proteins the density of PLA signals may be so high that it is impossible to discern the individual signals, see Figure 11. Duolink ImageTool can not quantify regions of merged signals; only separable signals can be counted. For images taken with fluorescence microscopy, a quality control function displays a warning if signals are merged. In this case the data is best analyzed by fluorescence intensity measurement or area fraction of sample with signal, using software for traditional fluorescence analysis. The primary antibodies used in the assay can be titered down to reduce the number and incidence of merged signals.

9. Troubleshooting

Some general guidelines are given below.

PROBLEM	PROBABLE CAUSE	SUGGESTED SOLUTION
No or few signals in positive samples	No or insufficient binding of primary antibodies	Optimize your primary antibodies individually with the Duolink In Situ single recognition approach, IHC or IF in positive control samples. Optimize parameters such as fixation, retrieval protocol, blocking and buffer conditions etc for the subsequent Duolink In Situ double recognition assay. Evaluate and optimize fixative, retrieval, incubation temperature, time, concentration and buffer composition.
	Insufficient reaction volume	Ensure that your reaction area corresponds to the reaction volume. The droplet must cover the reaction area. Encircle your reaction area using a hydrophobic barrier and incubate the slides in a humidity chamber to prevent evaporation. Do NOT use a cover slip to disperse the droplet.
	Mounting media	The intensity of the PLA signals will decrease quickly in some mounting media. Duolink In Situ Mounting Medium with DAPI is optimized for preserving the PLA signals.
	Wrong filter used for acquisition	The fluorophore in the Amplification Green has an excitation wavelength of 501 nm and an emission wavelength of 523 nm and can be detected using the same filters as for e.g. Cy2 or FITC. The fluorophore in the Amplification Orange has an excitation wavelength of 554 nm and an emission wavelength of 576 nm and can be detected using the same filters as for e.g. Cy3. The fluorophore in the Amplification Red has an excitation wavelength of 598 nm and an emission wavelength of 634 nm and can be detected using the same filters as for e.g. Texas Red. The fluorophore in the Amplification Far Red has an excitation wavelength of 644 nm and an emission wavelength of 669 nm and can be detected using the same filters as for e.g. Cy5. Make sure that you use appropriate filters.
	Inefficient ligation	Keep ligation incubation time and temperature. Ensure that no excessive amount of wash solution remains on slide before addition of ligation reagents. Ensure that the Ligase is active (i.e. has been kept at -20°C) and that correct dilution of the reagents have been used. Prepare fresh dilutions just before use; do not allow mix, with enzyme, to stand for more than 5 minutes before use.
	Inefficient amplification	If the signals are very weak they can appear to be few, as only a fraction reaches above detection threshold. Ensure that no excessive amount of wash solution remains on the slide before addition of amplification reagents. Keep amplification time and temperature. Ensure Polymerase is active (i.e. has been kept at -20°C) and that correct dilution of the reagents have been used. Prepare fresh dilutions just before use; do not allow mix, with enzyme, to stand for more than 5 minutes before use.

PROBLEM	PROBABLE CAUSE	SUGGESTED SOLUTION
High background – general cause	Incomplete deparaffinization	If you use paraffin embedded samples, incomplete removal of paraffin can cause background. Use fresh solutions if necessary and ensure that correct times during deparaffinization have been used.
	Custom blocking solution	If you have used your own blocking solution, allow primary antibody and PLA probes to be in contact with your blocking reagent before addition to the sample.
	Insufficient washing of slides	Ensure sufficient washing under gentle agitation, a minimum volume of 70 ml is recommended. Use fresh washing solutions and jars.
	Drying of sample	Ensure good humidity during all incubation steps and never let slides dry out after washes and before addition of reagents.
	Unspecific binding of primary antibodies	Titrate primary antibodies (IHC, IF or PLA) with respect to concentration, temperature, time and buffer and ensure appropriate fixation protocol was used.
Large amounts of non-amplification dependent background	Dust, salt or fixation precipitates cause highly fluorescent particles	Discard wash solutions and use new solutions and washing jars. If the problem remains, sterile filter all washing solutions. Wash your cells at least twice to ensure that the culture medium is removed before adding the fixative.
Fluorescent mist over entire slide	Ink-type pen has been used	Never label slides with ink-type pens. Use diamond or graphite pen.
	Inappropriate wash buffers	Use Duolink In Situ Wash Buffers A and B during the Detection protocol.
	Inappropriate mounting medium	Use Duolink In Situ Mounting Medium with DAPI for mounting of the slides.
	Contamination in wash solutions	Discard wash solutions and use new solutions and washing jars.
Red fluorescent nuclei	Inappropriate wash buffers	Use Duolink In Situ Wash buffer B in the final washing step after the Amplification.
	Inappropriate mounting medium	Use Duolink In Situ Mounting Medium with DAPI for mounting of the slides.
Large variation of signals over slide (individual reaction)	Uneven spread of reagents during different steps	Ensure that entire area of investigation is covered during each step.
	Partial drying of parts of the area during incubation	Check before each new step that no region has dried during incubation. If so, use a better humidity chamber.

PROBLEM	PROBABLE CAUSE	SUGGESTED SOLUTION
The reaction spreads over a too large surface/ is unable to cover sample	No or insufficient delimitation around reaction area	Use a grease-pen or alike to encircle reaction area.
	Inappropriate reaction volume	Adjust reaction volume according to your reaction area. For guidance on reaction volumes please refer to section "Reagent Preparation" or the separate Reaction Volume Guide.
No nuclear staining is observed	Mounting without mounting media containing DAPI	Ensure using Duolink In Situ Mounting Medium with DAPI.
	Wrong filter used for acquisition	Nuclear staining is performed with DAPI, excitation 360 nm and emission 460 nm. Correct filters must be used.

If problems remain, please contact us at support@olink.com or +46 18 444 3970

Appendix A – Wash Buffers formulas

DUOLINK IN SITU WASH BUFFER A

Alternative 1 – use Duolink In Situ Wash Buffer A (Art. no. 82047)

To prepare a 1x buffer, dissolve the content of one pouch in high purity water to a final volume of 1000 ml.

Store pouches at room temperature. Expiry date is marked on each individual lot. 1x solutions may be kept at room temperature for short time storage (one week or less). For long time storage store at +4ºC. Bring the solutions to room temperature before use.

Alternative 2 – make your own Duolink In Situ Wash Buffer A

Dissolve 8.8 g NaCl, 1.2 g Tris base and 0.5 ml Tween 20 in 800 ml high purity water. Adjust pH to 7.4 using HCl. Add high purity water to 1000 ml (final concentrations 0.01 M Tris, 0.15 M NaCl and 0.05% Tween 20). Filter the solution through a 0.22 µm filter and store at +4°C. Bring the solutions to room temperature before use.

DUOLINK IN SITU WASH BUFFER B

Alternative 1 – use Duolink In Situ Wash Buffer B (Art. no. 82048)

To prepare a 1x buffer, dissolve the content of one pouch in high purity water to a final volume of 1000 ml.

Store pouches at room temperature. Expiry date is marked on each individual lot. 1x solutions may be kept at room temperature for short time storage (one week or less). For long time storage store at +4ºC. Bring the solutions to room temperature before use.

Alternative 2 – make your own Duolink In Situ Wash Buffer B

Dissolve 5.84 g NaCl, 4.24 g Tris base and 26.0 g Tris-HCl in 500 ml high purity water. Adjust pH to 7.5 using HCl. Add high purity water to 1000 ml (final concentrations 0.2 M Tris and 0.1 M NaCl). Filter the solution through a 0.22 µm filter and store at +4°C. Bring the solutions to room temperature before use.

Appendix B – Duolink In Situ in chamber slides

The following protocol describes how to wash and fix cells that are placed on chamber slides, before using them in a Duolink In Situ assay. In order to avoid drying of the samples, prepare just a few slides at a time.

Culture and treat your cells in a container of your choice, e.g. flasks or chamber slides. Before fixation, cells should be plated in chamber slides and allowed to grow to desired confluency. Fixation should be done as soon as possible after harvesting the cells, in order to minimize degradation of proteins. If cells are grown too dense, it may be difficult to separate nuclei clusters in the final image analysis.

Washing before fixation

1. Pour out the cell culture medium by quickly inverting the chamber slides. Quickly add cold wash buffer of your choice, e.g. 1xPBS, so the wells are completely filled to prevent the cells from drying.
2. First wash: Take one slide at a time and pour out the wash buffer by inverting the slide. Fill up immediately with fresh wash buffer.
3. Second wash: Repeat step 2.
4. Third wash: Remove the chambers while the wash buffer remains in the wells. Do not let the cells dry out. If your slide contains a silicon barrier, remove it as well. Quickly place the slide in a Coplin jar with cold wash buffer and shake it manually for a few seconds.

Fixation

1. Transfer the slides from the Coplin jar with wash buffer to a new Coplin jar with a fixative of your choice, e.g. freshly thawed 3% PFA in 1xPBS. Fix the slides according to your protocol, preferably with gentle agitation.
2. Rinse three times with wash buffer, e.g. cold 1xPBS, 2 min per rinse (for PFA fixation, add 200 µl 1M glycine/Coplin jar in the second wash).
3. Wash quickly in MilliQ water once to remove salt.
4. Use the slides directly in your Duolink In Situ assay, or dry and store the slides at -20°C. When drying the slides, try to speed up the drying process before freezing in order to avoid degradation of proteins at room temperature.
5. Delimit the reaction area by using a grease pen before proceeding with the Duolink In Situ assay protocol.

Appendix C – Duolink In Situ on cover slips

Place the cover slips in a multiwell plate when running the Duolink In Situ assay. Mark the edges of the cover slip with a hydrophobic pen to delimit the reaction area. Use the Reaction Volume Guide (Doc. no. 80520) for guidelines on how much reaction volume is needed per reaction area.

Incubation steps

Make sure that the cells do not dry out and that the temperature is kept at 37°C during the incubation steps. In order to improve heat transfer and ensure an even temperature throughout the plate, use the Duolink In Situ Microplate Heat Transfer Block (Art. no. 82065-0001) pre-heated to 37°C.

Wash steps

Perform the washing steps with the cover slips still in the multiwell plate. Put the plate on a shaker during the washing steps, and add as much wash buffer as possible. Use the same washing times as recommended in the Duolink In Situ assay protocol. Make sure that the cells do not dry out when changing wash buffer.

Mounting

Mount each cover slip by turning it upside down on a regular microscope slide using a minimal volume of Duolink In Situ Mounting Medium with DAPI in between. More details are found in section 7.3, chapter 7, in the Duolink In Situ User Manual.

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Olink Bioscience
Dag Hammarskjölds v. 52B
SE-752 37 Uppsala, Sweden
www.olink.com